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Cyclic AMP-Related and Cation-Affected Human Platelet Chloride Transport Regulation

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Summary: Cystic fibrosis has been characterized as a defect in the regulation of cyclic AMP-dependent transepithelial chloride transport. The activation of cyclic AMP-dependent protein kinase A by cyclic AMP occurs normally in cystic fibrosis cells, but they fail to transport chloride ions in response to protein kinase A stimulation. Defective chloride secretion and abnormal electrolyte transport occurs in several organs including the lung, sweat glands, intestine and pancreas. The present work was aimed at exploring whether the same or similar regulatory systems are functional in platelets, and if they are altered or deficient in individuals with cystic fibrosis. Chloride transport in platelets from normal subjects and from cystic fibrosis patients was measured by cell sizing techniques where chloride permeability is the limiting factor. In platelets from healthy volunteers, the chloride channel blocker, 5-nitro-2-(3-phenylpropylamino) benzoic acid, inhibits the transport in a dose-dependent manner. The preservation of chloride transport capability is shown to be dependent upon the presence of either Ca^{2+} or two divalent cation substitutes, Cd^{2+} or Cu^{2+}. It is also shown that in normal subjects 0.1 μmol/l prostaglandin E_1, which elevates cyclic AMP 6 times and abolishes platelet aggregation, significantly enhances the rate constant of the transport. Furthermore, in five out of nine cystic fibrosis patients studied, platelet chloride transport did not respond to stimulation by prostaglandin E_1.

Introduction

Chloride transport in various cells is a topic of great interest. In platelets, it has been studied in the context of their volume-regulatory behaviour (1). When subjected to hypotonic NaCl medium (200 mosmol/l) platelets respond with a rapid swelling phase of 30 seconds followed by a volume decrease with a partial return toward the original volume (1). The reduction in volume in the second phase involves a loss of anions and potassium ions. The addition of the ionophore gramicidin to the medium induces a high permeability to both Na^+ and K^+, thus losses or gains of salts and the associated changes in volume are limited by the conductive anion permeability and provide its measurement (2). It has been shown (1) that platelets suspended in hypotonic NaCl medium in the presence of gramicidin react with continuing swelling. In hypotonic Na_2SO_4 medium, on the other hand, the initial swelling (osmotic equilibration – attributed to the inflow of water driven by the imposed osmotic gradient) is followed by a modest shrinkage. It was assumed that the platelet membrane is impermeable to SO_4^{2-}, with shrinkage driven by the outward chloride gradient. When Na^+ is replaced by K^+ (or Rb^+) the volume decrease is abolished and the rapid initial swelling phase is followed by a slower secondary swelling (1). As expected, no secondary swelling was observed if Na^+ was replaced by the impermeate cation N-methylglucamine. The rate of the secondary swelling in KCl medium is substantially enhanced by the addition

of gramicidin, suggesting that in swollen platelets potassium permeability is the limiting factor of the rate of swelling. When this limiting factor is removed by gramicidin, the swelling becomes limited by chloride permeability. The permeabilities of two additional anions – SCN^- and NO_3^- – behave like that of chloride, allowing a rapid secondary swelling at comparative rates, while citrate behaves like SO_4^{2-}. The present work extends the studies to the effect of divalent cations on the platelet chloride permeability.

Cystic fibrosis is the most common congenital disease among Caucasians. It is a disease of the secretory epithelia, tissues that mediate the transport of chloride, accompanied by sodium and water, between the blood and the lumen. The secretion requires activation of the secretory pathway by hormones or neurotransmitters, utilizing cyclic AMP or Ca^{2+}.

In recent years (1990–1992), the search for the basic defect in cystic fibrosis has reached a decisive stage since the identification of the responsible gene (3). It has been suggested that the 10pS gene product entitled cystic fibrosis transmembrane conductance regulator is either a regulator protein directing a chloride channel to the plasma membrane and conferring on it the ability to be regulated by cyclic AMP (4), or that it is a regulated chloride channel per se (5–7). Furthermore, the cystic fibrosis transmembrane conductance regulator has been purified to homogeneity and reconstituted into proteoliposomes in which it exhibited regulated chloride channel activity, providing evidence that the protein itself is the channel (8).

Platelet conductive ion fluxes exhibit similar features to those of epithelial cells (1), and are known to be responsive to hormones such as epinephrine, which is important in the regulation of fluid transport in the trachea and in sweat glands. Thus, the aim of the present work was to study chloride transport regulation in blood platelets, and to probe whether it is under cyclic AMP control.

Materials and Methods

Reagents and solutions

Gramicidin D, EGTA and prostaglandin E_1 were obtained from Sigma Chemical Co. (St. Louis, MO); 5-nitro-2-(3-phenylpropylamino) benzoic acid was a gift from Dr. B. Brauer from the Hebrew University of Jerusalem.

Acid-citrate-dextrose solution was composed of 65 mmol/l citric acid, 11 mmol/l glucose, and 85 mmol/l trisodium citrate. The *Tyrode* medium (modified), contained 137 mmol/l NaCl, 1 mmol/l KCl, 0.42 mmol/l Na_2HPO_4, 0.5 mmol/l $MgCl_2$, 5.5 mmol/l glucose and 20 mmol/l HEPES, pH 7.35, adjusted to 290 mosmol/l. Stock solution of gramicidin D (1 mmol/l) was prepared in dimethylsulphoxide.

Subjects

Assays in control platelets were performed with blood samples drawn randomly several times from ten healthy volunteers. Cystic fibrosis patients were from the Pediatric Cystic Fibrosis Clinic of the Soroka Medical Center, Ben-Gurion University Hospital in Beer Sheva. For all the children the diagnosis of cystic fibrosis was based on a sweat chloride concentration over 60 mmol/l and the presence of either pancreatic insufficiency or chronic obstructive pulmonary disease, or both. All the patients were under routine standard therapy including daily chest physiotherapy (by parents), high caloric diet with pancreatic enzymes and inhaled bronchodilators (β_2-agonists). Antibiotics were given according to sputum culture results (only when exacerbation of respiratory symptoms occurred). However, all children were without any acute symptoms during the time of the study. All patients and/or their parents were fully aware of the tests, so that the blood samples were taken only after informed consent was obtained.

Mutations analysis

The mutations were studied by Dr. *Bat-Sheva Kerem* and coworkers from the Hadassah Medical School, Hebrew University, Jerusalem, as previously described (9).

Preparation of platelet suspension

Venous blood was obtained from subjects who had not taken any medication during the preceding 10 days. The blood was collected in plastic tubes and anticoagulated with acid-citrate-dextrose at a volume ratio of blood : anticoagulant of 6 : 1. Platelet-rich plasma was obtained by centrifugation at 120 *g* for 10 min and had a pH of 6.5 ± 0.1.

Gel-filtered platelets were obtained by passing 0.5–0.8 ml of platelet-rich plasma through a Sepharose 2B column (5.5 × 0.76 cm), equilibrated with modified *Tyrode* medium. The platelets were eluted with the same medium.

Platelet volume and chloride permeability measurements

Volume measurements were conducted by electronic sizing using a Coulter counter, model ZF with a Coulter channelyzer C1000. Orifice diameter was 70 µm. An aliquot of the platelet suspension was diluted to a platelet count of $120 \cdot 10^6$/l. The mean cell volume was calculated from the volume distribution curves using polystyrene latex beads for calibration. To determine the conductive anion permeability as previously described (1), volume changes were measured in the presence of the ionophore gramicidin to induce a high permeability to Na^+ and K^+. As noted by *Grinstein* et al. (2), under these conditions, losses or gains of salts and associated changes in volume are limited by the conductive anion permeability of the cell membrane. The volume changes, therefore, provide a measure of anion permeability in general and of chloride permeability in particular. For the measurements, platelets were suspended in isotonic NaCl (*Tyrode*'s modified medium) for 7.5 minutes in the presence of 0.4 µmol/l gramicidin. On dilution with water to ⅔ of the original osmolarity, a very rapid, continuing swelling is observed, indicating chloride permeability. The rate constant of the permeability (relative volume change over time) was calculated as described (1). For the experiments designed to study the effect of divalent cations (figs. 2, 3 and 4) platelets were suspended in isotonic medium containing a mixture of NaCl and KCl *Tyrode* solutions (2 + 1, by vol) and the suspension was diluted with water as above. In figures 2 and 4, at the time points indicated, individual aliquots were separated and treated with 0.4 µmol/l gramicidin. The relative volume changes were measured by electronic sizing.

Other measurements

Cyclic AMP level in platelets was measured by a protein binding assay according to *Gilman* (10).

Platelet aggregation was assayed as previously described (11).

The final free Ca^{2+} concentrations and the concentration of the various divalent cations to be added to achieve a final free concentration of 0.15 µmol/l in the presence of 2 mmol/l EGTA were calculated by the software "Chelation 2", developed by Prof. *D. Chipman* from Beer Sheva, according to the dissociation constants of each of the cations with EGTA.

Results

In a previous paper (1), conditions were established for the evaluation of the conductive anion permeability in platelets. It is based on platelets suspended in isotonic NaCl medium in the presence of the ionophore gramicidin to induce a high permeability to cations. Upon induction of hypotonicity, gain of chloride is associated with volume increase, which is limited by the conductive chloride permeability. Thus, the extent of swelling under these conditions apparently provides a measurement of chloride permeability. Some characteristics of the platelet conductive anion transport were described (1).

If the system described indeed reflects conductive chloride permeability, then it is conceivable that an inhibitor of chloride channels – 5-nitro-2-(3-phenylpropylamino) benzoic acid – would inhibit the activity measured comparable to results in other cell types (12, 13). Figure 1 depicts the inhibitory profile of 5-nitro-2-(3-phenylpropylamino) benzoic acid upon platelet chloride permeability. The inhibition is detected at concentrations above 5 µmol/l and reaches a plateau of 80% inhibition above 20 µmol/l. Further characterization of the system is now provided.

The involvement of divalent cations

In the previous study (1), it was shown that platelets, suspended in an isotonic medium containing NaCl and KCl in a ratio of 2 : 1, upon induction of hypotonicity, react by initial osmotic swelling and do not change their volume for over one hour. Moreover, the maximal rates of the secondary swelling, initiated by gramicidin added at various times after the initial swelling, are remarkably similar. Figure 2 shows the involvement of Ca^{2+} in this gramicidin-induced swelling. When 2 mmol/l EGTA are added to the platelet suspension, the half-life of the decline of the chloride permeability is 30 ± 3 minutes (mean of 11 experiments ± SE). However, the addition of equimolar concentrations of both EGTA and Ca^{2+} (2 mmol/l) extends the capability of the process for at least 50 minutes. Figure 3 depicts the dependence of the permeability upon Ca^{2+} concentration. The concentration necessary to maintain half maximal activity ($c_{1/2}$) after 30 minutes in the hypotonic medium is 0.14 µmol/l. Is the effect of Ca^{2+} specific? This issue was addressed by the comparison of the effect of seven divalent cations of two different metal families: Mg, Ca, Sr and Ba of the earth alkali metals and Mn, Cd and Cu of the transition metals group. In the presence of EGTA

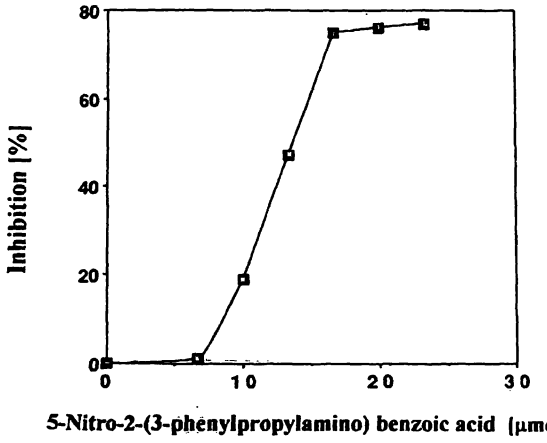

Fig. 1 Inhibition of the volume-induced chloride permeability by 5-nitro-2-(3-phenylpropylamino) benzoic acid.
Platelets (2×10^6) were suspended in 10 ml of isotonic *Tyrode* medium (modified) in which Cl^- is the predominant anion, supplemented with 0.4 µmol/l gramicidin. After 7.5 minutes the suspension was diluted with 5 ml of water to 200 mosmol/l and the relative volume changes were measured by electronic sizing (a representative Cl^- permeability sequence is depicted in fig. 5). 5-Nitro-2-(3-phenylpropylamino) benoic acid at the indicated concentrations was added 1 minute before the addition of the water.

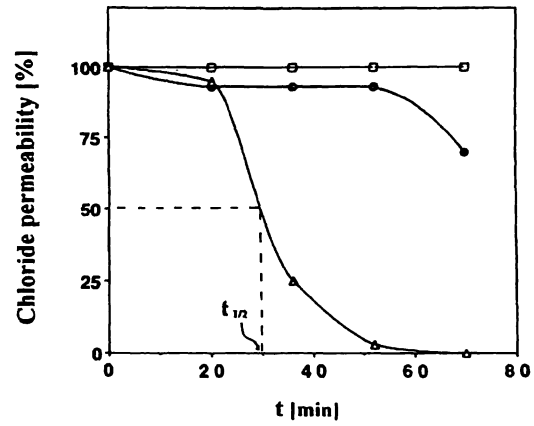

Fig. 2 Effect of EGTA and Ca^{2+} on the extent of chloride permeability with time.
Experimental conditions as in figure 1.
Gramicidin was omitted from the *Tyrode* medium. In parallel experiments, the medium was supplemented by either 1 mmol/l Ca^{2+} to serve as the control (□), 2 mmol/l EGTA (△) or 2 mmol/l EGTA and 2 mmol/l Ca^{2+} (●). Gramicidin was added to the controls and the tests at various time intervals after the induction of hypotonicity (zero time), and chloride permeability, measured by cell swelling, followed. The rate constants obtained with EGTA only or EGTA and Ca^{2+} at each time interval were compared with the corresponding control designated as 100%.

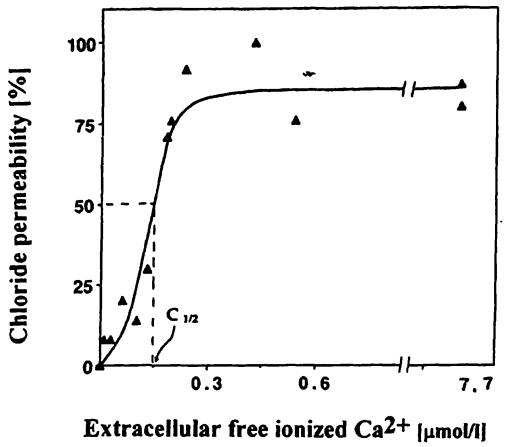

Fig. 3 The effect of extracellular free Ca^{2+} concentration on the extent of chloride permeability.
The experimental design was as described in figure 2. EGTA and Ca^{2+} were added to the *Tyrode* medium to gain the final free Ca^{2+} concentrations as indicated. These were calculated by the software "Chelaton 2" (Methods). Gramicidin was added 30 minutes after the induction of hypotonicity.

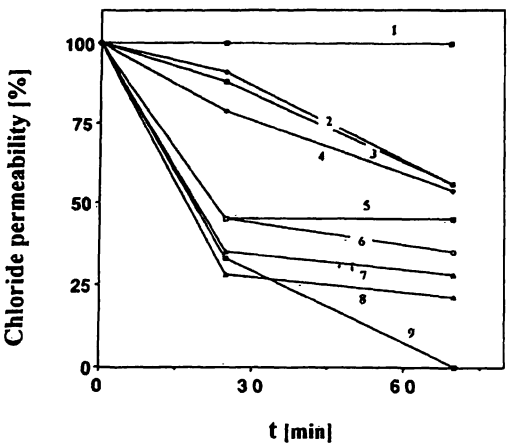

Fig. 4 The effect of divalent cations on the extent of chloride permeability with time.
Experimental conditions as in figure 3 with 2 mmol/l EGTA and appropriate concentrations of the cations to produce final free concentrations of 0.15 µmol/l each. The calculation of the concentrations to be added was carried out with the software "Chelation 2" (Methods).

1. control (1 mmol/l Ca^{2+})
2. Cd^{2+}
3. Cu^{2+}
4. Ca^{2+}
5. Mn^{2+}
6. Ba^{2+}
7. Mg^{2+}
8. Sr^{2+}
9. 2 mmol/l EGTA

and at a final equal concentration of the free cation of 0.15 µmol/l, Ca^{2+}, Cd^{2+} and Cu^{2+} apparently show the same "protective" effect while the other four fail to fulfill the need for the cation (fig. 4). This, of course, may result from different (lower) affinities for Mn^{2+}, Ba^{2+}, Mg^{2+} and Sr^{2+}.

The impact of cyclic AMP

Since chloride channels in several cells are known to be regulated by cyclic AMP levels (14–16), and since prostaglandin E_1 is known to elevate cyclic AMP concentrations (17), the effect of prostaglandin E_1 on platelet swelling limited by chloride permeability was studied. Figure 5 depicts an accelerated chloride permeability in the presence of 0.1 µmol/l prostaglandin E_1. Table 1 summarizes the results of ten similar experiments showing a statistically significant 44% enhancement of the apparent platelet chloride permeability caused by prostaglandin E_1. Is the effect of prostaglandin E_1 mediated by cyclic AMP? Two lines of evidence suggest a positive answer. First, table 2 demonstrates that under the conditions of the experiments described in figure 5 and table 1, cyclic AMP concentration is indeed elevated six fold by prostaglandin E_1. Secondly, ADP-induced platelet aggregation, known to be inhibited by elevated cyclic AMP concentrations, was totally abolished by as little as 0.02 µmol/l prostaglandin E_1 (data not shown).

Of particular interest is whether the system described has any relevance to the expression of cystic fibrosis. In view of the fact that it has been shown that the defect in cystic fibrosis patients is not the activity of the chloride channel but rather its response to control mechanisms such as an increase by cyclic AMP-dependent protein kinase (15, 18), the response of platelet chloride transport to prostaglandin E_1 in cystic fibrosis patients was evaluated (tab. 3). Nine patients were probed. Their rate constants of chloride permeability did not differ from those of healthy volunteers. However, five of them did not respond to prostaglandin E_1 (as compared to a minimum response of 15% augmentation in all the control

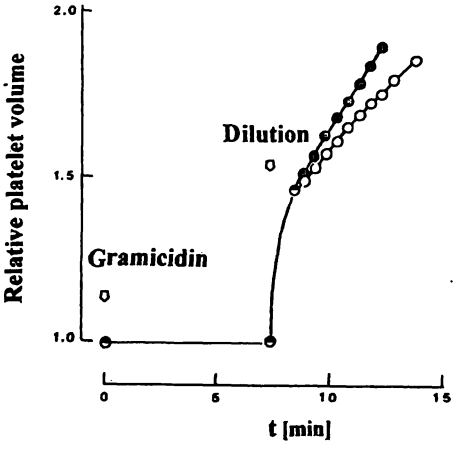

Fig. 5 Effect of prostaglandin E_1 on chloride permeability in control platelets. Experimental conditions – as described for figure 1. Prostaglandin E_1 0.1 µmol/l (solid circles) or its solvent – 1 ml ethanol (open circles) were added at the time of dilution. Representative of ten experiments.
Temperature was 25 °C.

subjects studied, summarized in tab. 1), but four did respond normally to the prostaglandin.

Tab. 1 Enhanced volume-induced chloride permeability by prostaglandin E_1

Treatment	Rate constant min^{-1}
Control	0.109 ± 0.008
Prostaglandin E_1, 0.1 μmol/l	0.157 ± 0.011

The experimental conditions were as in figures 1 and 5.
Results are means of ten experiments ± S.E.
The stimulatory effect of prostaglandin E_1 is statistically significant according to the *Student*'s t test, $p < 0.01$. Neither of the individuals studied responded by less than 15% augmentation.

Tab. 2 Effect of prostaglandin E_1 on platelet cyclic AMP concentration

Treatment	cAMP pmol/10^9 platelets
Control	2.8
Prostaglandin E_1, 0.1 μmol/l	17.0

Platelet-rich plasma was incubated with either ethanol (1 ml/l) or prostaglandin E_1 (0.1 μmol/l) for one minute, followed by centrifugation and aspiration of the supernatant.
Cyclic AMP in the pellet was extracted by boiling for 5 min in 50 mmol/l acetate buffer pH 4.0.
The results are means of duplicates.

Tab. 3 Response of volume-induced chloride permeability of cystic fibrosis patients to prostaglandin E_1

Patient	Sex	Age (a)	Genotype paternal/maternal	Response to prostaglandin E_1*, ratio of the rate constants
M.M.	♂	2.5/12	G542X/G542X	1.63
A.Y.	♂	2	N.A.**	1.55
M.Y.	♂	2	DF508/?	2.00
E.R.	♀	3	N.A.**	1.35
F.E.	♂	9	DF508/W1282X	1.00
P.A.	♂	9	W1282X/DF508	1.00
S.S.	♂	13	3849 + 10kbC→T	1.00
S.Y.	♂	14	3849 + 10kbC→T	1.00
H.B.	♂	17	DF508/DF508	1.00

The experimental conditions are as in figure 1.
Each patient was assayed versus an age- and sex-matched healthy volunteer.
At least four assays were carried out from a given patient's blood sample.
Two of the patients were assayed under two different occasions with the same results.
* The values express the stimulation of the rate constant caused by 0.1 μmol/l prostaglandin E_1 as compared with the vehicle (aqueous ethanol, volume fraction 0.7)
** Not available.

Discussion

Conductive platelet anion permeability, mainly chloride permeability, is relatively low in isotonic medium and increases substantially on hypotonic swelling (1). This fact was used in the present work to study the control of this conductive feature in healthy people as well as in cystic fibrosis patients. To further support the interpretation that the assay previously developed (1) does indeed measure chloride transport, inhibition by a chloride channel blocker, 5-nitro-2-(3-phenylpropylamino) benzoic acid (12, 13), is documented, which adds to the previously observed inhibition by oligomycin C (1), shown to inhibit chloride pathways in lymphocytes (19). However, the results of *Haws* et al. (20) of "little or no inhibition" of cystic fibrosis transmembrane conductance regulator channels in immortalized human airway cells by 100 μmol/l 5-nitro-2-(3-phenylpropylamino) benzoic acid prevent us from drawing an unequivocal conclusion.

The dependence of the chloride permeability upon specific divalent cations indicates that there are at least two factors which affect chloride transport in platelets; one is catabolic – associated with cyclic AMP, and the other is concerned with cations. Another possibility, as shown for epithelial cells (21, 22) and epithelial apical membrane (23), is that Ca^{2+} and cyclic AMP activate different chloride channels (23). Other options – such as the change in Ca^{2+} concentrations resulting in altered cytoskeleton and subsequent swelling – are still open.

Apical chloride channels are opened and chloride secretion is stimulated by a variety of hormones and neurotransmitters that increase intracellular levels of cyclic AMP (24, 25). The same regulatory pattern is now principally demonstrated in platelets. Prostaglandin E_1 enhances cell swelling limited by chloride permeability, an effect that correlates with the elevation of cyclic AMP levels and with the inhibition of platelet aggregation well known to occur upon increased cyclic AMP concentration (17). Nevertheless, it cannot be ruled out that cyclic AMP alters platelet cytoskeleton, changes intracellular Ca^{2+} concentrations or activates transporters, resulting in a relative volume change.

In cystic fibrosis patients, the main molecular defect has been identified as chloride impermeability in various epithelia (26–30). In fact, hormonal secretagogues do stimulate cyclic AMP accumulation appropriately, but the chloride channels fail to open (26, 31). To study the pathophysiology of the disease at the biochemical-physiological regulatory level in platelets, the effect of prostaglandin E_1 on platelet chloride conductive permeability in cystic fibrosis patients was probed. The well known heterogeneity in the clinical presentation of cys-

tic fibrosis, such as the involvement or absence of pancreatic insufficiency (32), and the variation in the severity of the disease in the whole community (33) have been partially attributed to different mutations at the cystic fibrosis locus. Studies of the correlation of cystic fibrosis mutations with pancreatic function, meconium ileus, liver involvement, lung disease and gender have been reported and reviewed (34, 35). Relevant examples are:

(a) remarkable correlation between ΔF508 homozygosity (ΔF508/ΔF508) and pancreatic insufficiency ("severe" mutation) versus a genotype of ΔF508/non-ΔF508 or non-ΔF508/non-ΔF508 that have a mutant cystic fibrosis product that confers a sufficient phenotype (36);

(b) a severe disease, reflected by pancreatic insufficiency, high incidence of meconium ileus, early age at diagnosis, poor nutritional status and variable pulmonary function, is associated with homozygosity for the W1282X mutation and the heterozygosity for the ΔF508 and W1282X mutations (9). The variability is intriguingly reflected in the patients studied by us as well. Of the nine tested, five patients lacked the regulatory response to prostaglandin E_1 stimulation, whereas the other four were responsive. Although the number of patients is limited, it is notable that in agreement with the examples described above, neither of the four responding patients exhibits the "severe" mutations, while three out of the five non-responders possess the severe mutations (F. E., P. A. and H. B.) while the other two carry the rare mutation 3849 + 10kbC → T which is not yet well characterized. Along with the fact that the chloride channel response to cyclic AMP-dependent protein kinase is defected in cystic fibrosis patients rather than the activity itself, there is good evidence that some mutant cystic fibrosis transmembrane conductance regulators are defectively processed (37) and that the very activity of the chloride channel is altered (38). Such mutations have been implicated to be associated with the mild-disease-form. Thus, it may also be suggested that the four responding patients in the present study belong to this group of mutations. It is interesting to point out that the non-responsive patients in the present study were all above nine years old, while the responsive ones were aged three years or less. Such a phenomenon could theoretically be understood if cystic fibrosis were an acquired disease but not a congenital one. Yet, the majority of the patients are diagnosed by the time of adolescence. This fact may suggest that although hereditary, some of the molecular manifestations of the disease are expressed at a later stage of life. It might be that at an early stage some of the components of the chloride transport complex are still capable of leaking adequately from the endosomal membranes to the functional sites in the plasma membrane. At later stages of life they may become arrested for numerous reasons, as yet unclear. Such a possibility adds interest to the study of the system, but it does not allow the use of the assay of prostaglandin E_1 enhancement of platelet chloride conductive permeability as a reliable diagnostic tool for cystic fibrosis. It could, however, be used as a tool to follow the development of the disease. The main advantage of the platelet system is that it serves as an easily available, simple assay system to further study characteristics of the chloride transport system in health and disease.

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